Review Article

Optimization of chitosan-based scaffold technology in tissue engineering: A review

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ABSTRACT

This article presents ordered and systematized data obtained from modern literary sources devoted to the use of chitosan in tissue engineering. At the end of the article, data from our scientific research is presented, in which an attempt was made to create a three-dimensional scaffold based on chitosan. Sterile scaffolds were subcutaneously sewn into the soft tissues of the withers area or in the area of artificial femoral defects of rats. The tissue sections together with the implants were removed 4 and 8 weeks after placement under the same conditions. When removing, the mobility of the regenerants *in situ*, the state of the surrounding fiber, the presence of vessels feeding the scaffold, the severity of the adhesive process, and the degree of biodegradation of the scaffolds were evaluated. In the field of bone defect replacement, attention was paid to the degree of osseointegration, the completeness of the closure of the defect, and the density of the regeneration. The analysis of modern world literature and the results of our experiments show that the main components of the innovative trend and the use of chitosan for tissue engineering of articular cartilage are: modification of chitosan scaffold by copolymerization with various organic compounds; improvement of methods for the preparation of three-dimensional biomimetic nanostructured chitosan scaffolds; intensification of the use of chitosan-based scaffolds in the process of creating them to improve their viscosity-strength, chondroinductive, and antibacterial properties of biologically active additives.

Keywords: Tissue engineering, Scaffold technologies, Chitosan, Cartilage tissue, Hip joint

Introduction

The high rate of development of biomedical technologies in the field of medical effects on damaged cartilage is due to a whole range of socio-economic, medical, and general biological factors [1, 2]. In particular, an increase in life expectancy is accompanied by a prolongation of an active lifestyle among the elderly. As a result, the frequency of joint damage similarly increases [3, 4]. At the same time, there remains a need for a high quality of life even in the presence of various diseases [5, 6]. In addition to age-related changes in joints, it is impossible not to take into account the frequency and degree of injury to large joints associated with human professional activity [7].

The development of medical materials and technologies allows the use of predominantly minimally invasive arthroscopic

Access this article online	
Website: www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Bulovina M, Derevyankina A, Khadzhibekova A, Khoshbekyan A, Babayan M, Bochkarev M, et al. Optimization of chitosan-based scaffold technology in tissue engineering: A review. J Adv Pharm Educ Res. 2024;14(3):18-25. https://doi.org/10.51847/WKRwRsIEaB

technologies for the treatment of injuries and chronic joint diseases [8].

At the same time, it must be understood that articular cartilage has an initially low ability to recover. Therefore, in most cases, during treatment, it is necessary to replace the lost structures and functions of articular cartilage and, at the same time, stimulate their cells to remodel the defect replacement zone into full-fledged cartilage tissue [9, 10].

Currently, autogenous chondroplasty is considered the best option for repairing damaged cartilage. However, this approach has several unavoidable limitations and disadvantages, and does not provide adequate restoration of full-fledged joint function for a long time. Many modern researchers believe that tissue engineering technologies will become the next leader in this field in the next decade [11-13].

The essence of joint tissue engineering consists of the development and manufacture of bioengineered scaffolds (scaffolds) and their subsequent implantation to the patient to compensate for the defect and stimulate regeneration of the damaged three-dimensional tissue structure [14]. The key problem is to ensure consistent and complete remodeling of the tissue-engineered structure into its cartilage. This requires predictable control actions on the processes of colonization, proliferation, differentiation, and adequate phenotypic expression of cells in the substance of the scaffold and the future matrix of its cartilage. One of the key approaches to such management is the planning and manufacture of a scaffold with a predetermined set of these properties [15].

To date, there is a fairly wide range of materials suitable for the manufacture of scaffolds. The main requirements for these materials include their absence of cytotoxicity, their ability to elicit an inflammatory and immune response, their ability to maintain adhesion, fixation, proliferation, and differentiation of cells, their bio-resorbability through conventional metabolic pathways, the availability of self-healing abilities, and their ability to change their structure and properties in response to environmental factors, including mechanical stress [16, 17].

One of the promising natural materials that are becoming increasingly recognized in the formation of tissue engineering structures and possessing most of the above properties is modified chondroitin sulfate (chitosan), a deacetylated form of the chitin polymer widely distributed in nature [18].

The purpose of this scientific work is to substantiate the relevance of the use of chitosan for tissue engineering of articular cartilage based on data from modern scientific literature and our research.

Advantages of chitosan-based scaffold technologies in cartilage tissue engineering

A group of Italian scientists led by R. Muzzarelli has been actively studying the potential of chitosan use as a material in regenerative biomedicine since 1988 [19-21]. These researchers successfully applied chitosan scaffolds to replace defects in the dura mater, wound surfaces, and fibrous cartilage, noting the subsequent full-fledged morphological restoration of defects without any functional disorders. The authors believe that the beginning of the use of chitosan to restore lost supporting tissues has opened a new milestone in tissue engineering [22, 23].

The availability of raw materials for the production of chitosan and the ease of improving its physicochemical properties by enzymatic treatment make chitosan a very promising basis for the design of modern scaffolds. In addition, chitosan is biomimetic to the cartilage's own matrix, non-toxic, and has full biocompatibility, bio-resorbability, and moderate antibacterial properties. Pronounced chondro- and osteo-inductive effects of three-dimensionally organized chitosan have also been determined [24, 25].

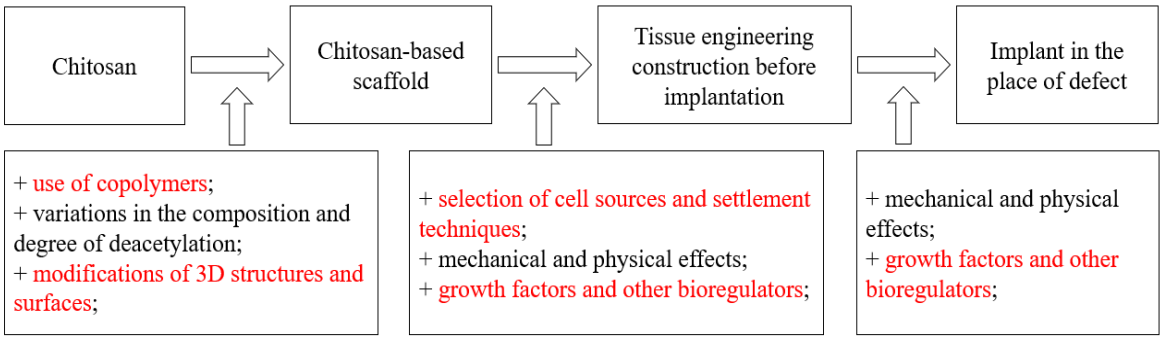
Chitosan scaffolds have a high ability to induce cell migration, adhesion, proliferation, and induction of the necessary chondral or osteogenic phenotype, as a result of which intensive remodeling of bone and cartilage tissue is ensured, while the resorption of surrounding tissues is not activated [26].

Chitosan has found application in surgical and orthopedic dentistry in the treatment of fractures, distraction osteogenesis, treatment of osteomyelitis, and osteoporosis. In maxillofacial implantology, when titanium implants were coated, chitosan reduced the severity of the reaction of surrounding tissues to surgery and contributed to accelerated osseointegration of implants [23].

When creating chitosan scaffolds, it is possible to implement a three-dimensional (3D) porous structure with a certain pore size and the thickness of the partitions between them. It has been empirically established that the restoration of cartilage tissue requires porosity values of the order of 80-85%, a pore diameter of the order of 150-400 microns, and a thickness of the partitions between them of at least 50-70 microns [21]. This is necessary to ensure a certain strength, high cell adhesion, and the possibility of transporting gases and metabolites in newly formed tissues [23].

Initially, the high viscosity of chitosan solutions allows the use of various methods for creating porous scaffolds: lyophilic drying, and foaming with gases. The hard-elastic properties of some chitosan modifications proposed for use in tissue engineering approach the values peculiar to spongy bone and can withstand a compression load of about 75 MPa with pore sizes of about 250-500 microns [27, 28].

Figure 1 shows a systematization of the numerous approaches used by specialists to improve the effectiveness of chitosan-based scaffold technologies. The most active efforts of researchers are currently focused on the modernization of chitosan-based scaffold technologies by varying copolymers, switching to nanostructured products, and connecting depots of growth factors.



Note: the areas of active transition to nanomaterials and nanotechnology are highlighted in red

Figure 1. Critical processes affecting the final quality of scaffold technology in the restoration of damaged articular cartilage

Modifications at the stage of chitosan production. Copolymerization

The chemical activity of chitosan allows for various modifications of the polymer with a wide range of biologically active components of both organic and inorganic composition. Chitosan copolymerizes well with organic polyacids, alginate, polyglycols, gelatin, and proteins. It should be noted that when chitosan interacts with modifying agents, its biologically active properties are not only not lost, but in some cases, they are enhanced [29, 30].

Chitosan initially has moderate antimicrobial activity. This is due to the presence of active binding sites for surface toxins, bacterial antigens, and effects on other components of their cell wall. These properties can be purposefully enhanced by copolymerization with acyl residues of organic acids, ligands of various organic antiseptics, organometallic compounds (Au, Ag, Cu, Ti, and Pt), and surfactants [31-33].

Chitosan-polylactide scaffold was copolymerized with microspheres of amide-polylactic acid in hexane-diamine-propanol. One of the ways to form high-quality cartilage tissue is to modify the chitosan surface using a porous elastomer made of poly-L-lactide-caprolactone.As the wettability improves the cellular compatibility of the scaffold increases without significantly changing its physical properties [34, 35].

Some scientists have determined the degree of influence of hyaluronic acid inclusions with different molecular weights on chondrogenesis from mesenchymal stem cells cultured on a spongy chitosan scaffold [36].

The creation of a chitosan-based gel scaffold made it possible to obtain a liquid polymer under normal conditions, but taking the form of sol at temperatures close to body temperature. This became possible after the creation of the chitosan–glycerophosphate sodium – hydroxyethylcellulose composite [37]. It was determined that chondrocytes in the reconstructed cartilage are able not only to survive but also to retain their ability to secrete the matrix [38].

The processes occurring in cartilage and the subchondral zone of bone after the remodeling of micro-defects using an implant based on the composition chitosan - glycerol phosphate - whole blood were analyzed [39]. For a long time, a large number of osteoclasts were observed in the remodeling zone, while the bone beams were structurally integrated [40].

The composite matrix chitosan-polybutylene-succinate was obtained by pressing followed by leaching [41]. Thus, matrices of different porosity with variable pore size were obtained.

Manufacture of nanostructured scaffolds

In the last decade, many different methods of manufacturing three-dimensional biomimetic scaffolds have been developed for the needs of tissue engineering. These include electrospinning, phase separation, freeze-drying, and self-assembly [42].

The principle of the electrospinning technique is that under the action of high voltage, repulsive forces are formed in capillary tubes filled with a viscous polymer solution, initiating jets of outflow from the capillaries [43]. The conservation of repulsive forces between the jets eventually leads to the formation of the thinnest (nanoscale) polymer filaments, which are collected in a special collector [44, 45]. In this case, the thickness of the filaments can vary due to variations in viscosity, electrical conductivity, and surface tension of the solution, as well as technological conditions (hydrostatic pressure in the capillary tube, electric field strength, distance between the probe and the collector) [46].

Phase separation can be induced thermally or by precipitation techniques, and is used for the manufacture of porous membranes or foamed materials [47]. Compared with electrospinning, phase separation has a better potential for the manufacture of three-dimensional nanofiber scaffolds with a more uniform porous structure [48].

Freeze drying is an integral part of the technology for converting soluble labile materials into sufficiently solid stable structures, initially in the food industry, pharmaceuticals, and enzyme industries [49]. This technology (lyophilization) includes three main stages: freezing of the solution at a sufficiently low temperature (about -70 ° C); transfer of frozen samples to a chamber where the pressure is reduced to several millibars. Some of the water is removed at this stage (direct sublimation); but most of the water is removed by desorption at the third stage of final drying [50, 51].

Biologically active additives that promote adhesion and proliferation of chondrocytes occupy a special place in the

creation of chitosan-based scaffolds for cartilage remodeling and repair [52]. It is known that such a powerful biologically active substance as insulin causes chondral differentiation. Malafaya *et al*. [53] paid attention to chondrogenic differentiation and the development of cellular systems that provide the synthesis of biomolecules for its stimulation. To do this, various forms of insulin were added to the chitosan scaffold used as a potential model system for cartilage and cartilage tissues. It has been shown that the insulin dose in the system (5%) is the most effective in stimulating chondrogenic differentiation.

Kuo and Wang [54] demonstrated cartilage regeneration in hybrid scaffolds consisting of polyethylene oxide and chitosan with the addition of CDPGYIGSR peptide. The pores, with an average diameter of about 200-250 microns, were interconnected and evenly distributed. The high percentage of polyethylene oxide in the matrix contributed to an increase in the strength of the pore walls. It has been proven that this peptide promotes the adhesion of chondrocytes and accelerates their proliferation, in addition, the addition of this peptide promotes the synthesis of type 2 collagen.

Results and Discussion

Our team attempted to create a three-dimensional scaffold based on chitosan. Chitin, the starting material for chitosan, was obtained from the outer skeleton of crustaceans of the genus Pandalus, by washing with tap water, followed by treatment with a 10% $NaHCO_3$ solution in the presence of surfactants [55]. After settling, repeated washing of the finished product were carried out. After that, it was demineralized, finally rinsed, and dried to a dry-air state [56].

Chitosan was obtained by deacetylation from chitin, previously ground to a size of 1-2 × 2-3 mm. Carrying out the process in a vacuum of a water jet pump contributed to a significant decrease in the concentration of oxygen in the reaction zone, the presence of which, as is known, increases the degree of destruction of chitin [57, 58]. The filtered chitosan was a highly hydrated product with a water content of more than 70%. To prevent keratinization, chitosan was dried in a thermostat at 35.0–40.0 °C to a dry-air state [59, 60].

To assess the quality of the chitosan obtained, the indicators included in the technical specifications for food chitosan were used: appearance, color, taste, and smell [61]. Porous 3D matrices based on chitosan were created using the original freeze-drying method [62, 63]. 10 sterile matrices were created from the original chitosan and chitosan produced by InFood LLC (Russia). The properties of the matrices were tested in vivo in experiments using 24 white male Wistar rats weighing from 180 g to 240 g. The experimental protocol complied with the ethical standards set out in the International Code of Medical Ethics (1994), the Rules of Laboratory Practice (GLP), and European Community Directives 86/609EEC [64]. The data obtained were processed using methods of variational statistics using the statistical packages "Statistics for Windows" v.6.0 and Biostat (version 4.03).

The three-dimensional scaffold created by the team of scientists was based on chitosan, the physical and chemical properties of which are shown in **Table 1**. It can be noted that the resulting drug fully complies with the technical conditions for the main parameters and corresponds to the data obtained by other researchers [65-67].

Table 1. Physical and chemical properties of chitosan

Indicator	The norm for technical conditions	Sample
Mass fraction of moisture, %	≤ 10.0	9.6
pH of 1% solution in 2% CH_3COOH	≤ 7.5	4.2
Degree of deacetylation, %	≥ 80%	92%

In the first series of experiments, sterile scaffolds measuring 5×5 mm **(Figure 2)** were subcutaneously sewn into the soft tissues of the withers area of 10 rats under aseptic conditions. In the second series, smaller fragments of the scaffold were implanted in the area of artificial femoral defects (channels with a diameter of 1.5 mm and a depth of 3 mm). The tissue sections together with the implants were removed 4 and 8 weeks after placement under the same conditions.

Figure 2. The appearance of a porous chitosan scaffold

When removing soft tissue samples, the mobility of the regenerants *in situ*, the state of the surrounding fiber, the presence of vessels feeding the scaffold, the severity of the adhesive process, and the degree of biodegradation of the scaffolds were evaluated. In the field of bone defect replacement, attention was paid to the degree of osseointegration, the completeness of the closure of the defect, and the density of the regeneration [68]. The material was fixed in formalin and after rapid alcohol wiring and complete dehydration, it was enclosed in paraffin through xylene [69]. After dewaxing, paraffin sections 5-7 microns thick were stained with hematoxylin and eosin, the tricolor Masson method, and picrotoxin Van Gieson [70]. As a result, evidence has been obtained for the formation of a full-fledged cartilage regeneration in place of the chitosan scaffold **(Table 2)**.

Table 2. Volume fractions of tissue elements in the regenerate at the site of implantation of chitosan-based matrices to rats (%, M±m)

Indicator	Control	The timing of the experiment	
		4 weeks	8 weeks
Regenerates during heterotopic implantation			
Chitosan fragments	0	39.6±2.6	8.2±0.4
54.2±Cartilage tissue	0	14.7±1.2	54.2±3.
Connective tissue	45.6±2.8	34.2±2.4	32.1±2.4
Vessels	5.3±2.4	11.3±0.9	6.6±2.8
Adipose tissue	48.7±2.7	0	0
Regeneration during orthotopic implantation			
Chitosan fragments	0	24.3±1.2	5.6±0.4
Cartilage tissue	97.6±4.2	44.8±1.1	73.5±5.8
Connective tissue	2.1±0.3	24.3±1.6	17.2±1.4
Vessels	0	5.8±0.6	2.8±0.4

The following stages of modification of chitosan-based tissue-engineered scaffolds are supposed to be carried out along the path of increasing the biocompatibility and biodegradation of chitosan, which will require the use of new modifying agents and using the capabilities of immunohistochemical methods for analyzing tissue remodeling.

Conclusion

Thus, the analysis of modern world literature and the results of our experiments show that the main components of the innovative trend and the use of chitosan for tissue engineering of articular cartilage are: modification of chitosan scaffold by copolymerization with various organic compounds; improvement of methods for the preparation of three-dimensional biomimetic nanostructured chitosan scaffolds; intensification of the use of chitosan-based scaffolds in the process of creating them to improve their viscosity-strength, chondroinductive and antibacterial properties of biologically active additives.

Acknowledgments: All the authors were involved in the conceptualization, methodology, formal analysis, writing, and editing of the manuscript.

Conflict of interest: None

Financial support: None

Ethics statement: The protocol for experiments with laboratory animals complied with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

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